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A long-range interaction in Q_β RNA that bridges the thousand nucleotides between the M-site and the 3′ end is required for replication

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ABSTRACT

The genome of the positive strand RNA bacteriophage Q_β folds into a number of structural domains, defined by long-distance interactions. The RNA within each domain is ordered in arrays of three- and four-way junctions that confer rigidity to the chain. One such domain, RD2, is about 1,000-nt long and covers most of the replicase gene. Its downstream border is the 3′ untranslated region, whereas upstream the major binding site for Q_β replicase, the M-site, is located. Replication of Q_β RNA has always been puzzling because the binding site for the enzyme lies some 1,500-nt away from the 3′ terminus. We present evidence that the long-range interaction defining RD2 exists and positions the 3′ terminus in the vicinity of the replicase binding site. The model is based on several observations. First, mutations destabilizing the long-range interaction are virtually lethal to the phage, whereas base pair substitutions have little effect. Secondly, in vitro analysis shows that destabilizing the long-range pairing abolishes replication of the plus strand. Thirdly, passaging of nearly inactive mutant phages results in the selection of second-site suppressor mutations that restore both long-range base pairing and replication. The data are interpreted to mean that the 3D organization of this part of Q_β RNA is essential to its replication. We propose that, when replicase is bound to the internal recognition site, the 3′ terminus of the template is juxtaposed to the enzyme's active site.

Keywords: evolution; *hfq* gene; host factor; phage RNA; Q_β replicase; RNA structure

INTRODUCTION

The single-stranded RNA coliphage Q_β contains a genome of about 4,000 nt encoding three structural proteins and a replicase (Fig. 1A). The replicase assembles with elongation factors EF-Tu, EF-Ts, and ribosomal protein S1 to form the holoenzyme. With the additional help of host factor (HF), the product of the *Escherichia coli hfq* gene (Muffler et al., 1997), the holoenzyme can copy the plus strand into a minus strand. Synthesis of plus strands from minus strands only requires EF-Tu and EF-Ts as cofactors (reviewed by Blumenthal & Carmichael, 1979). Efficient multiplication of Q_β RNA can be performed in vitro with purified components and Q_β replicase is the best characterized RNA-dependent RNA polymerase known today.

Much work has been invested to understand the basis of template selection. The enzyme has great specificity and only recognizes Q_β RNA and other members of the group III and the group IV phages, such as SP and NL95, but not the more distantly related group I and II phages, like MS2, nor does the enzyme copy any of the many bacterial RNAs it encounters during infection (Yonesaki et al., 1982; reviewed in van Duin, 1988).

As a first step to examine this problem, the binding sites of the holoenzyme (-HF protein) on Q_β RNA were characterized by a variety of techniques (Meyer et al., 1981; Miranda et al., 1997). The surprising conclusion from these experiments was that there are two internal binding sites far away from the 3′ end. The 3′ end was not bound by the holoenzyme. A new question therefore was how the 3′ end of the RNA is brought to the reactive center of the enzyme. For some time, one of the options was that the auxiliary HF protein would provide this activity. Indeed, electron microscope pictures on binary complexes between Q_β RNA and Hfq protein showed double-loop structures, in which the 3′ terminus appeared folded back to the two internal binding sites (Barrera et al., 1993).

Recently, the question of enzyme–template recognition was greatly simplified when Q_β was adapted to an *hfq* null mutant (Tsui et al., 1994; Schuppli et al., 1997). Most suppressor mutations that evolved destabilized

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[1]Dedicated to Valdis Berzins, Biomedical Research Centre, University of Latvia, Riga. Deceased April 19, 1998.

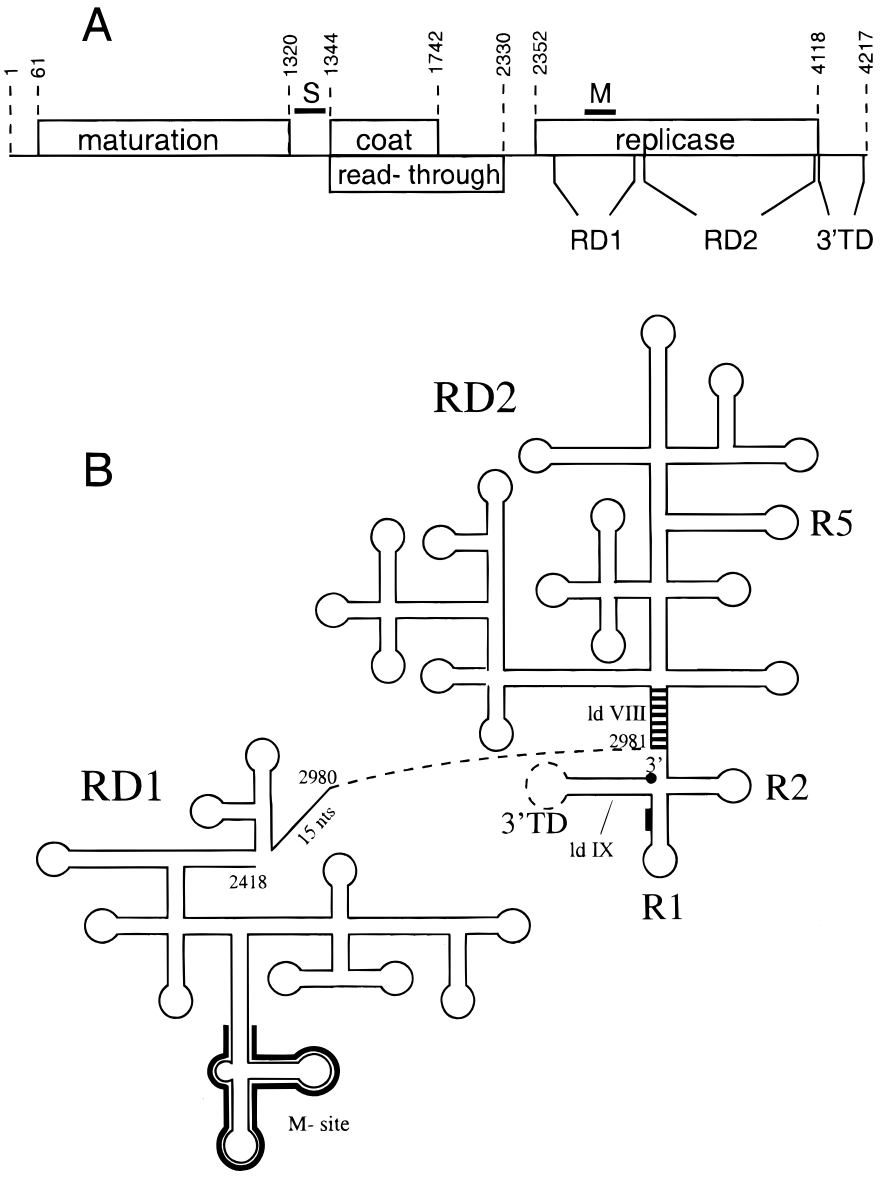

FIGURE 1. A: Genetic map of the single-stranded RNA coliphage Q_β. Binding sites S and M of the replicase holoenzyme are indicated by black bars. Location of replicase domain 1 and 2 (RD1, RD2) and of the 3′-terminal domain (3′ TD) are indicated. The maturation protein is also involved in cell lysis. **B:** Schematic view of the RNA folding in the last three domains of Q_β RNA. Each stem and stem-loop shown here is also present in the detailed model, but they are not drawn to scale. Bulges and internal loops have been omitted for simplicity. R1, R2, etc. identify the separate stem-loop structures. The 3′ UTR or 3′ TD is shown only as a dotted circle. The full 2D structure of this part is given in Figure 8. The detailed structures of RD2 and RD1 and their derivation are given by Beekwilder et al. (1995) and Beekwilder (1996), respectively. ld VIII stands for long-distance interaction VIII. Its full structure is given in Figures 3 and 8. The black rectangle in R1 indicates the end of the replicase reading frame. The very 3′ terminus of the RNA is marked by a black dot. Base pairs are only indicated for ld VIII.

local hairpins in the 3′-terminal domain, but they also weakened a long-range base pairing that masked the 3′-terminal C residues (ld IX in Fig. 1B). The observations show that, as far as the phage is concerned, HF is a dispensable protein that probably acts by increasing the accessibility of the 3′ terminus of the RNA to the replicase. This description fits with a recent finding that the translation of the *E. coli rpoS* messenger becomes HF independent if inhibitory structures at the ribosome binding site are destabilized (Brown & Elliott, 1997). In both instances, weaker secondary structures can substitute for HF.

Of the two internal replicase binding sites, one is located in RD1 in the replicase-coding sequence around position 2750 (M-site), whereas the other is found at the start of the coat protein gene (S-site) (Fig. 1A). A further simplification of the system came with the observation that the first 2,400 nt of Q_β RNA, including the S-site, are not required for synthesis of the minus strand (Miranda et al., 1997). The S-site is thought to

only allow replicase to compete with ribosomes at the major ribosome entry site (Weber et al., 1972).

But even in this simplified scheme where the host factor and more than half of the RNA have been removed from the scene as dispensable for replication, the molecular basis of 3′ end binding is not self-evident, nor is what provides the template specificity. One possibility raised in the past is that the folding of Q_β RNA is such that replicase binding to the M-site positions the 3′ end of the RNA in the active site of the enzyme (Meyer et al., 1981). If this is to happen with great efficiency and speed, a prerequisite is that the structure of Q_β RNA brings together the M-site and the 3′ end.

Recently, we and others have presented secondary structure models for Q_β RNA based on electron microscopy, computer prediction, comparative analysis, and structure probing (Jacobson, 1991; Jacobson & Zuker, 1993; Beekwilder et al., 1995, 1996). An important feature of some of these models is indeed the presence of progressively longer-range interactions that restrict the flexibility of the chain, bring distant parts together, and are expected to result in a well-defined surface where the distance of the 3′ end to other segments of the RNA is fixed. The available structure models are therefore consistent with the possibility that the active site of the M-site-bound replicase is already positioned at the 3′ terminus rather than having to await its arrival by a chance event if the chain were highly flexible. The availability of an in vitro replication system of infectious Q_β cDNA clones and of RNA structure models provides us with the opportunity to start testing these ideas about enzyme–template recognition.

In Figure 1B, we show a simplified version of the Q_β RNA secondary structure covering nt 2418–4217 (the 3′ end). We distinguish three domains, RD1, RD2, and the 3′-terminal domain (3′ TD), coinciding with the 3′ UTR. The M-site lies within RD1 and is marked with a heavy black outline. RD1 and RD2 are connected by 15 nt, 8 of which are involved in pseudoknot formation by pairing to the loop of the 3′-terminal hairpin (not shown; manuscript in prep.). This interaction restricts the freedom of RD1 with respect to RD2.

It is clear that most of the contraction in this part of Q_β RNA is conferred by the long-range interactions. Thus, if our assumption is correct that the M-site and the 3′ end are brought together by structure, we expect that disruption of long-distance interaction VIII (ld VIII) will have a strong negative effect on replication (Fig. 2). (The same would also be true for disrupting ld IX or R1 or R2, but here we only concern ourselves with ld VIII.)

Our approach is to distort the base pairing in the proposed ld VIII by mutations in either side and assay the consequences for phage viability and for replication in vitro. In a second step, phages with a distorted ld VIII are passaged and suppressor mutations identified. If the structure model and our predictions are correct, second site suppressor mutations are expected to show

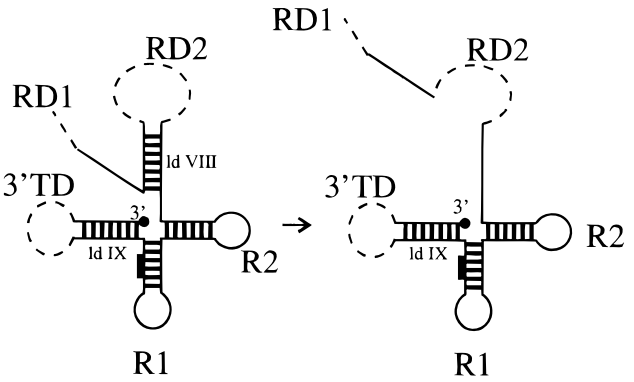

FIGURE 2. Further simplified view of the Q_β RNA structure around the 3′ terminus. Disruption of ld VIII, R2, R1, or ld IX is expected to change the position of the M-site with respect to the 3′ terminus.

up ≈ 1,000 nt away at the other side of ld VIII. Such pseudorevertants are then assayed for improvements in replication vis à vis the starting mutant. We here report that ld VIII is a key structure necessary for Q_β RNA replication.

RESULTS

The system

The complete sequence of Q_β is present on a plasmid under the transcriptional control of the T7 promoter. Mutations of our choice are introduced in Q_β cDNA and cells are transformed with this plasmid. When the wild-type sequence is present in a host devoid of T7 RNA polymerase (M5219), this results in about 10^9 phages in the supernatant of 1-mL overnight culture (Taniguchi et al., 1978). When T7 polymerase is present (BL21 (DE3)), this number rises to 5×10^{10} (Table 1). These two strains do not carry F pili and therefore cannot be reinfected by the phage. Sometimes we used F^+ strains expressing also T7 RNA polymerase as host of the

TABLE 1. Infectivity of various Qβ cDNA constructs.

	Bacterial strains used		
Plasmids	M5219 (F^-)	BL21(DE3) (F^-)	JM109(DE3) (F^+)
pT7Qb	1×10^{9a}	5×10^{10}	+
pT7S1	0	10	+
pT7S2	0	0	+
pT7S3	0	2×10^3	n.d.
pT7S4	0	0	0
pT7D1	0	0	+
pT7D2	0	7×10^5	n.d.
pT7R5	4×10^9	1×10^{10}	n.d.
pT7S1.1	0	1×10^4	n.d.
pT7S1.2	0	4×10^4	n.d.

[a]Plaque-forming units per mL overnight culture.

infectious plasmid to increase the capacity of the assay system to produce phages.

Long-distance interaction ld VIII is required for viability of Q_β

In Figure 3 (top panel), the structure of ld VIII is given. Both sides of the stem contain replicase-coding sequences and we have marked the codon wobble positions with an asterisk. Because the wobbles are not juxtaposed, it is not possible to change a base pair for another while maintaining the encoded amino acids. Comparison with related phages showed that the amino acids encoded by the 5′ side of ld VIII are conserved, whereas this is less so for the 3′ side. Thus, we decided to introduce our mutations in the wobble positions on the 5′ side and in the juxtaposed nucleotides on the 3′ side (first codon position). The six mutants whose performance and properties we analyzed are shown in Figure 3.

Construct S2, whose only differences with wild type are three wobble replacements, does not produce any plaques in the two F^- strains used (Table 1). Similarly S4, containing substitutions in the 3′ side of ld VIII, did not result in any phage production. Most surprising, however, when we combine the mutations of S2 and S4 to yield D2, we get 7×10^5 pfu in strain BL21. Therefore, the finding that S2 and S4 do not produce phage seems not due to the mutations per se, but to the fact that they disrupt the pairing. This is the first strong suggestion that ld VIII exists and that it is an important structural element in Q_β RNA. In accordance herewith, we find that constructs S1 and S3 in which ld VIII is less seriously distorted than in S2 and S4 do give rise to a modest amount of plaques in BL21 (Table 1). The data indicate that the less the structure is disrupted (S3), the less affected is its capacity to form plaques. The only mutant that we cannot yet explain is D1. This mutant combines the changes present in S1 and S3, but it does not produce phage in the two standard systems in spite of the base pairing. It seems that replacement of three A-U by 3 G-C pairs can make ld VIII too strong (see Discussion).

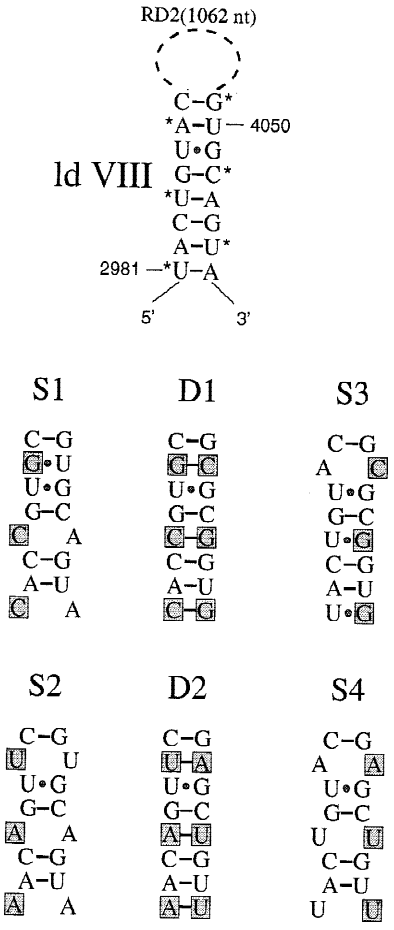

FIGURE 3. Base pairing scheme in ld VIII (top panel). Codon wobble positions of the replicase reading frame are indicated by asterisks. Lower panel shows the six mutants that were made. Grey boxes show replaced nucleotides.

Evolution of ld VIII mutants

If ld VIII is important to the phage, we may expect to find pseudorevertants from, for instance, S2, in which base pairing is restored by suppressor mutations in the 3′ side of ld VIII.

One practical problem here was that, e.g., construct S2 did not produce any phage even in BL21 cells, expressing the T7 polymerase. This plasmid was therefore transfected to an even more sensitive host expressing both the T7 polymerase and F pili, which allow reinfection (JM 109 (DE3)F^+). In such a system, the accidental creation of a single viable phage may be detected. This approach was successful. Measuring the titer in this assay system is not very meaningful; therefore, we have only indicated a (+) in Table 1 when phages were found in the bacterial lysates. In revertants from S3 and S4, the mutations introduced by us reverted back to the wild-type bases at one or two positions and these replacements are thus not very informative. Construct S1, however, yielded three revertants with a second site A → G suppressor mutation, more than 1,000 nt away from the initial mutation, precisely in ld VIII, where it restored base pairing (S1.1, Fig. 4). Similarly, two of the plaques derived from construct S2 showed an A → U second site suppressor mutation that also repaired the ld VIII stem (Fig. 4, S2.1). The further evolution of revertants S1.1 and S2.1 proceeded in a predictable way. It is noteworthy that the substitutions in S1.1 and S2.1 both lead to amino acid replacements not found in any natural isolate. These revertants then provide very strong evidence both for the existence and importance of ld VIII.

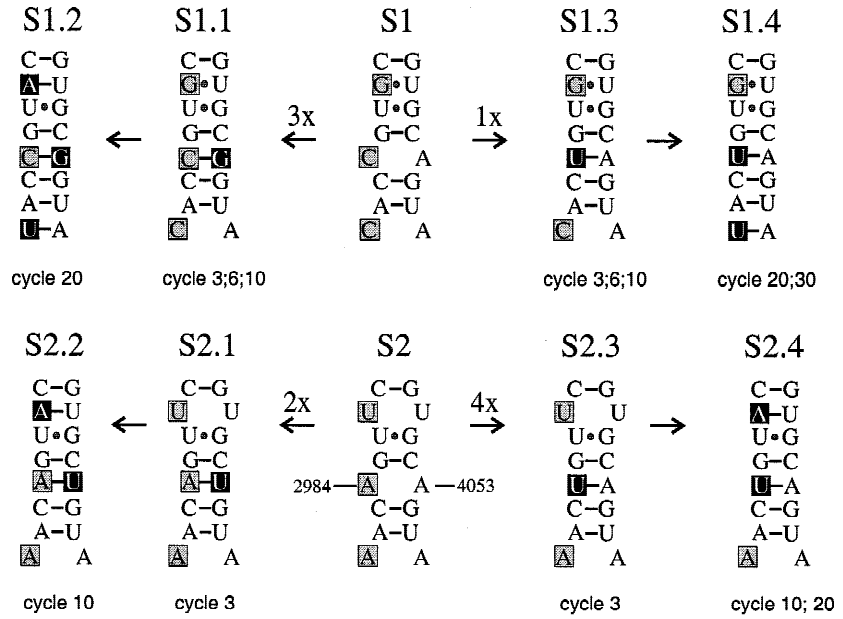

FIGURE 4. Structure of starting mutants S1 and S2 and their revertants. Grey boxes show the initial mutations introduced by us. Black boxes mark suppressor mutations after the indicated number of infection cycles. Numbers above arrows show how many times the specific revertant was found. In S2, two nucleotide numbers are given for convenience.

Distortion of Id VIII affects replication of Q_β RNA

It was surprising to find that, e.g., S2, containing only three wobble substitutions, had a titer that was more than 10^9 times lower than wild type (Table 1), the more so because only one transversion, A2984U or A4053U (Fig. 4), would suffice to yield a measurable titer (S2.3 and S2.1). It is assumed that the reduction in titer of a mutant construct is roughly proportional to the probability of creating a viable revertant by chance mutations (Olsthoorn & van Duin, 1996). Thus, if a transversion has a probability of 10^{-5} to occur per nucleotide per replication, we would expect construct S2 to give about 10^5 pfu/mL. Because we did not find any plaques in our standard host, this suggested that the pool from which revertants must be selected is extremely small, in turn indicating that the mutations interfere with replication.

To test this possibility, we prepared in vitro wild-type and mutant Q_β RNA transcripts from the corresponding T7 plasmids using T7 RNA polymerase. Then these transcripts were used for a single round of replication in vitro using pure Q_β replicase and HF. The results are shown in Figure 5.

It is clear that mutant Q_β RNAs with a distorted Id VIII are very poor substrates for Q_β replicase, whereas D2, having the three reversed base pairs, performs quite well. Interestingly, D1, containing a stabilized Id VIII, is also deficient in replication (see Discussion). The inability of mutant constructs to produce phages must thus, in all probability, be ascribed to a defect in replication. As an additional control, we also prepared T7 transcripts from revertants S1.1 and S1.2 after introducing their respective suppressor mutations in Q_β cDNA. As can be seen from Figure 6, the suppressor mutations that have accumulated in S1.1 and S1.2 progressively increase the capacity of the template to be replicated. Thus, as expected, these mutations are selected on the basis of improved replication.

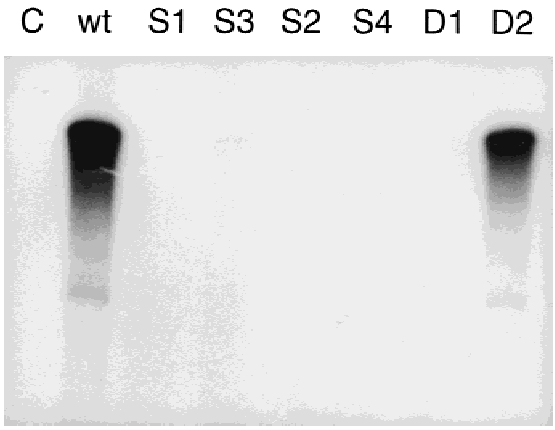

FIGURE 5. Minus-strand synthesis in vitro from wild-type and mutant Q_β RNA. Shown is an autoradiogram of ^{32}P-labeled RNA after electrophoretic separation in agarose gels under denaturing conditions. In the first lane C, no template was added. We measured rates of initiation as described by Barrera et al. (1993). The RNA, together with holoenzyme and HF, was incubated for 45 s at 37 °C in the presence of only GTP and ATP. Then polyethylene sulfonate (PES) was added to inhibit de novo initiation. Next, CTP and UTP were supplied to allow completion of minus strand. See Materials and Methods for further details. If overexposed, the autoradiograph shows weak bands of less than 1% of wild type for the inactive mutants.

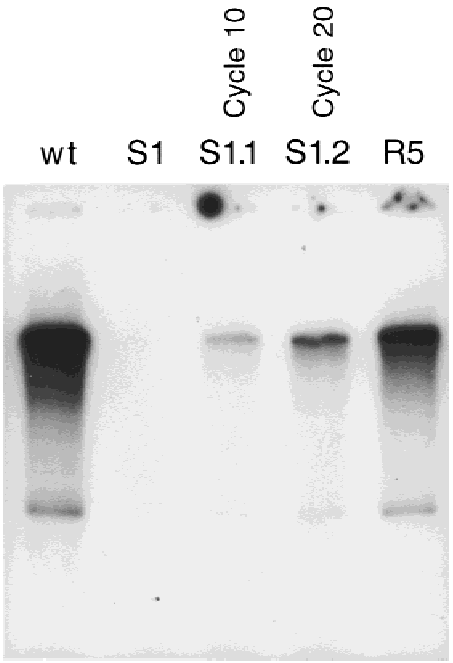

FIGURE 6. Replication of wild-type, mutant, and revertant Q_β RNA in vitro. S1.1 and S1.2 are successive revertants of S1. R5 contains three mutations in peripheral helix R5.

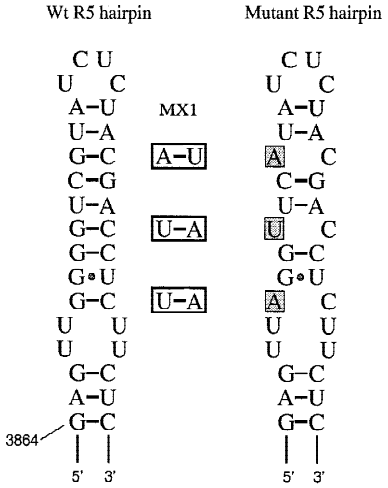

FIGURE 7. Structure of hairpin R5 and a mutant derivative. Covariations with the related phage MX1 are shown in boxes.

Replication is not impaired by distortion of a local hairpin

The results so far provide strong evidence that replication is very sensitive to the paired state of ld VIII. A further interpretation of this finding requires that we show that this effect is specific for ld VIII and that disruption of pairing in peripheral hairpins in the same domain is much less harmful. Accordingly, three base pair-disrupting substitutions were introduced in hairpin R5 (Figs. 1B, 7). We choose R5 because there is strong phylogenetic support for its existence as evident from the covariations with the MX1 sequence (Fig. 7). The three substitutions do not change the meaning of the codons and destabilize R5 in much the same way as the mutations in S2 and S4 destabilize ld VIII. As can be seen in Table 1, these three mutations hardly affect the capacity of the infectious clone to form plaques (pT7R5). In agreement, the capacity of the R5 mutant to be replicated in vitro is not strongly affected, in contrast to the results of the S1 mutant (Fig. 6, lanes 2 and 5). These experiments show that it is specifically the disruption of the long-distance interaction that abolishes replication.

DISCUSSION

In this paper, we show that a long-distance interaction in Q_β RNA (ld VIII), spanning more than 1,000 nt, is required for replication, more specifically for plus strand copying. The evidence is threefold. First, disruption of the base pairing decreases the titer of the infectious clone by about 10 orders of magnitude. When base pairing is restored by combining two mutants (D2), in themselves inactive, the ability to form plaques is largely recovered. Second, when mutants with a distorted ld VIII are passaged, second site suppressor mutations are selected that map at the side opposite the one where the mutations were introduced and that restore complementarity, even though these compensatory changes lead to new amino acids in the replicase. Third, mutants that did not or hardly produce plaques could not be replicated in vitro, whereas the revertants they produced could.

These results show that ld VIII is required for replication. Because neither ld VIII nor its constituent sequences have ever been implicated in replicase binding, the data suggest that ld VIII is necessary to bring the 3′ terminus in the vicinity of the M-site. In more general terms, the 3D structure of this part of the RNA appears essential for replication. We envisage that Q_β RNA is folded to position its 3′ end in the active center of the M-site-bound enzyme. Breaking up this folding by disrupting a major element disorients the 3′ end. This view may also bear on our understanding of the specificity of replication. As mentioned, Q_β replicase will only replicate closely related RNAs, but not that of phage MS2 nor any cellular RNA. This specificity cannot be explained by a sequence-determined recognition between template and enzyme, because contact between holoenzyme and template is mediated by the host-coded S1 subunit (Barrera et al., 1993; Brown & Gold, 1995a), a protein with a general preference for single-stranded polypyrimidine sequences (Subramanian, 1983; Bycroft et al., 1997). In the uninfected cell, this protein mediates messenger binding to ribosomes (Boni et al., 1991). According to all we know, ribosomal protein S1 is also a subunit of the MS2 replicase. Thus, it

seems more realistic to attribute specificity to the spatial fit between enzyme and template as initially advocated by Meyer et al. (1981). The specificity is thus not conferred by the proteins, but by the shape of the template. We think this paper presents the first structural data to directly support this concept for the RNA phages.

Thermodynamic stability of ld VIII is tuned

Our results show that when ld VIII becomes weaker, the potential to yield plaques goes down (S3 > S1 > S2). A simple way to account for these results would be to consider that ld VIII is essential and that, the less stable the structure, the less the fraction of molecules in which pairing exists at any one time. If so, the prediction is that structures more stable than wild type will perform at least as good as wild type. Things seem more complex, however, because this turns out not to be true. Mutant D1 in which ld VIII is more stable than in wild type showed no replication in vitro (and no plaques in vivo). With some effort, we have been able to recover a few revertants from this mutant. Four were sequenced and all had the penultimate G-C pair (2987–4050) changed to the weaker G·U pair. This structure was satisfactory as judged by the fact that it sustained a high level of phage production and remained unchanged for at least 20 cycles (not shown). The explanation for the nonviability of D1 seems therefore that ld VIII also can be too strong. Note that the new amino acids that we introduced in D1 do not affect the conclusion because the replication assay was performed in vitro with wild-type replicase. The finding that revertant S1.2 performs worse than wild type in in vitro replication (Fig. 6) may also be due to a stronger ld VIII. In S1.2, a U-A pair has been replaced by C-G. It is unlikely that ld VIII is an obstacle during elongation, because much stronger stems have been found not to stop replication (Klovins et al., 1997).

The requirement for a well-tuned stability suggests an outline for how replication starts. First, replicase binds the internal M-site. As a result, its active site is now juxtaposed at the terminal nucleotides, and these must be liberated from ld IX. As we have seen, this may be the task of the host factor because, in *hfq* mutants, Q_β evolves an accessible 3′ end. In this scenario, the tuning of ld VIII contributes in the following way. If ld VIII is unstable, the 3′ terminus never gets near the replicase tethered at the M-site but, if ld VIII is too strong, the terminus, although in the right position, cannot be recruited from the structure.

It is not immediately clear how the stability of ld VIII could influence the release of the 3′ end. However, if one envisages that the 3′ terminus is located in the heart of the quasi four-way junction, it seems possible that destabilizations in any of the four arms, R1, R2, ld VIII, ld IX, may promote its exposure. This view is supported by a recent analysis (Schuppli et al., 1997). When Q_β was passaged through an *hfq* null mutant, it adapted by a number of suppressor mutations. Almost all HF-independent phages analyzed carried a mutation destabilizing ld IX (Fig. 8). Some, however, carried additional mutations and two of these destabilized either ld VIII or R2 (Fig. 8; in R2 the stable tetraloop UACG is replaced by UACA). Thus, it seems possible that mutations in ld VIII and R2 can affect the accessibility of the 3′ end.

From this and the work of Brown and Elliott (1997), it appears that the absence of HF can be compensated by a weaker target structure. If HF is a destabilizer of RNA structure, and if ld VIII needs a balanced stability for maximal replication, the prediction is that mutants in which ld VIII is weakened will replicate even worse in the presence of HF. This is indeed what we find. Replication of destabilized mutants S2 and S3 is 5–20-fold inhibited by HF, whereas mutant D2, having about the same stability as wild type, is stimulated by the factor (Fig. 9).

Why is the 3′ terminus of the RNA phages base paired?

It is intriguing why the 3′ end of the RNA phages is so carefully hidden in base pairing when RNA replication seems best served with an accessible end (Biebricher & Luce, 1992; Brown & Gold, 1995b). Indeed, in many plant RNA viruses, the terminal nucleotides are not paired (Mans et al., 1991; van Rossum et al., 1997). If pairing was forced by creating complementarity, the template function seemed strongly reduced (Deiman et al., 1998).

We suppose that the differential terminal structure in pro-and eukaryotic viral RNA is related to a basically different status that free 3′ ends have in pro- and eukaryotic cells. In prokaryotic cells, accessible 3′ termini tend to be polyadenylated, which, in turn, is the signal for degradation (Xu et al., 1993; Haugel-Nielsen et al., 1996). Another part of the answer may be that, in bacteriophage RNA, switching from the translation to the replication mode requires some communication between ribosomes and replicase (Weber et al., 1972). A freely accessible 3′ end would not create a point of competition in prokaryotes. It might do so in eukaryotes, where the 3′ end of viral RNA also plays some role in translation (reviewed in Deiman & Pleij, 1997).

The need for organizing the template structure

Recently, Brown and Gold (1995a, 1996) presented an interesting study on the workings of Q_β replicase. The authors show the presence of two RNA anchoring sites on the holoenzyme, one on ribosomal protein S1, the other on EF-Tu. The Tu-site is used to copy the minus strand and any of the small RNAs generated during Q_β infection. Plus strand Q_β is the special case because it

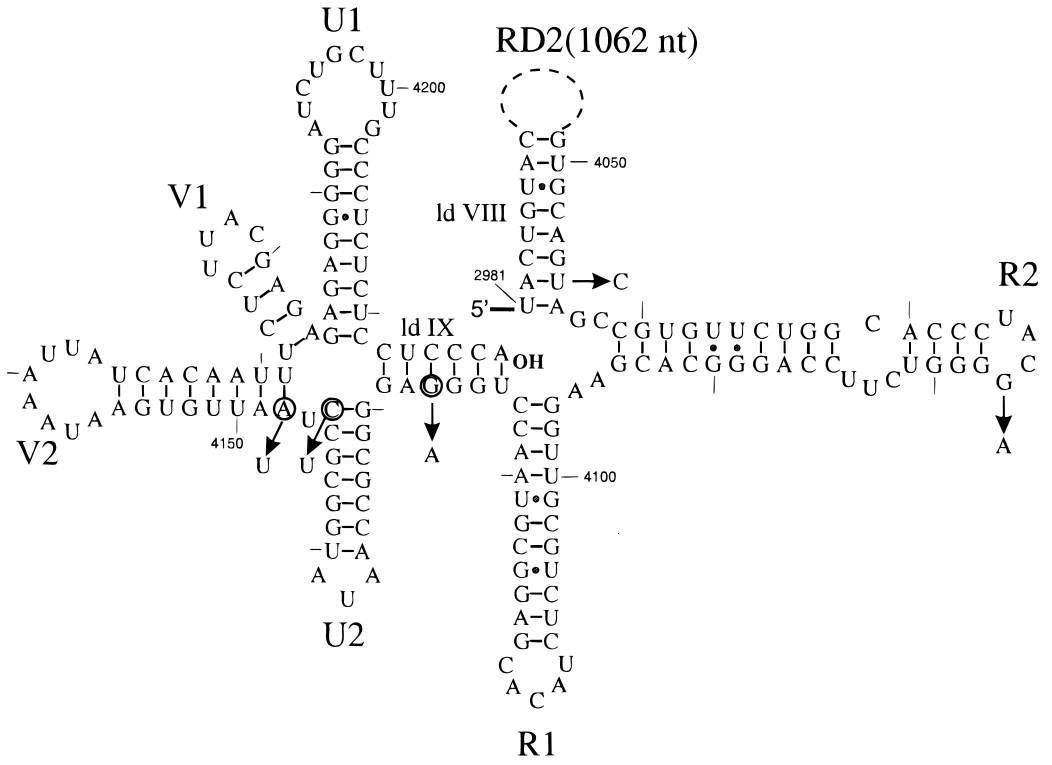

FIGURE 8. Secondary structure of Q_β RNA around the 3′ terminus. Base substitutions occurring in Q_β adapted to an *hfq* null mutant are shown. The encircled substitutions at positions 4127, 4146, and 4148 are found in all pseudorevertants. Those at positions 4077 and 4055 are found only in some.

is the only template that requires S1 to dock the replicase. Brown and Gold (1995b) show that an RNA needs only two features to be replicated: a sequence that anchors the replicase via S1 or EF-Tu, and a 3′ terminus ending in three consecutive C residues. Replication then starts when the active site of the replicase encounters the 3′ end by chance. True as this may be, we have to keep in mind that it is one thing to be a replicatable RNA, but another to be a successful RNA virus. Accordingly, the picture of replication that we present is somewhat more complex. A freely accessible 3′ end is likely to fall prey to RNA degradation and, in real life, it is unlikely that the encounter of the 3′ end of the template with the active center of the replicase is left to chance. Competition will probably select the RNA structure that wastes the shortest possible time between being captured by the replicase and being replicated. This goal seems best served by structuring the RNA in such a way that its 3′ end is positioned in the enzyme's active center at the initial binding step. At the same time, this proper juxtaposition of the essential template elements provides the stereospecificity needed to compete with an abundance of host RNAs present in the infected cell.

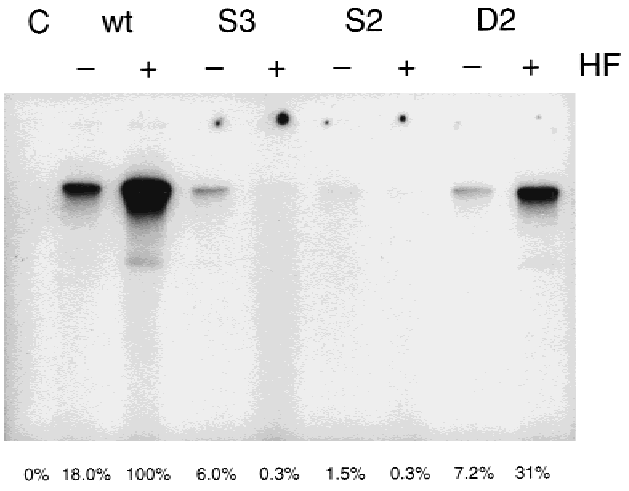

FIGURE 9. Differential effect of HF on replication of template with a wild-type and a destabilized ld VIII pairing. C stands for control in the absence of template RNA. Percentages are obtained by Betascope analysis. For further details, see legend to Figure 5.

MATERIALS AND METHODS

Bacterial strains and plasmids

Plasmids containing full-length Q_β cDNA and derivatives were grown in *E. coli* strain M5219 (*M72 lacZ*$_{am}$, *trpA*$_{am}$, *Sm*^{r}/*λbio*$_{252}$, *cI*$_{857}$, Δ*H1*) and DH5α (*supE*$_{44}$, Δ*lacU*$_{169}$ (ϕ_{80}, *lacZΔM15*), *hsdR*$_{17}$, *recA1*, *endA1*, *gyrA*$_{96}$, *thi-1*, *relA1*) at

37 °C. Generation of mutant phages from plasmids was performed in *E. coli* F$^-$ strain BL21(DE3) (*hsdS*, *gal* (λ*cIts*$_{857}$, *ind1*, *Sam7*, *nin5*, *lacUV5-T7 gene 1*) and F$^+$ strain JM109(DE3) (*thi*, Δ(*lac-proAB*), [*traD36*, *proAB*, *lacIqZ*Δ*M15*], λ$^-$, *lacUV5-T7 gene 1*), which carry the IPTG-inducible T7 RNA polymerase gene on their chromosomes. Cultures were grown without IPTG at 37 °C. For evolution of phages, *E. coli* K12 strain GM-1 (*ara*, Δ(*lac-proAB*), *thi Sm*^{r}, *F*$^+$) was used. All strains were grown on LC broth. Parent plasmid pBRT7Qβ, used for all mutagenesis, was a generous gift from Dr. H. Weber (Zürich). The plasmid contains full-length Q_β cDNA (plus strand) under control of the phage T7 RNA polymerase promoter (Barrera et al., 1993). This plasmid produces a titer of 10^9 pfu/mL culture after overnight growth in M5219 cells.

Generation and evolution of mutant Q_β phages

Mutant sequences were obtained by PCR-directed site-specific mutagenesis using overlapping mutant oligonucleotides (Ho et al., 1989). The mutant PCR products obtained were digested with *Bsi*W I and *Apa* I for the 5′ fragment, Q_β nucleotide numbers 2845 and 3680, respectively, and *Apa* I and *Spe* I for the 3′ fragment. *Spe* I is located in the vector (position 4226). We then exchanged with the corresponding fragments in plasmid pBRT7Q_β. Because most of the mutant plasmids were not able to form viable phages in cells without T7 polymerase, they were transformed to BL21(DE3) or JM109(DE3) cells. Transformants were inoculated in 2 mL LC containing 50 μg/mL ampicilin and grown overnight at 37 °C. Appropriate dilutions of the supernatant were plated on lawns of GM-1 cells (cycle 2). Individual plaques were picked and amplified in liquid cultures of GM-1 cells (cycle 3). Subsequent evolution of mutants was performed by serial transfers of 10^4 phages from the previous to the next cycle, as described (Olsthoorn et al., 1994). One milliliter of lysate was taken for phage RNA isolation after the appropriate number of cycles.

RNA isolation, RT-PCR, and sequence determination

Phages were precipitated from 1 mL of lysate by adding 330 μL of 40% polyethylene glycol (PEG) 6000 (Merck) in 2 M NaCl. After centrifugation, the pellet was suspended in 100 μL of RNA Insta-Pure buffer, and the RNA was extracted as recommended by the supplier (Eurogentec). Finally, the precipitated RNA was dissolved in 20 μL of bi-distilled water. Four microliters of this solution was used for reverse transcription with 1 U of AMV reverse transcriptase (Promega) using primer Bio303 complementary to the 3′ end of Q_β RNA (nt 4192–4217). The cDNA obtained was amplified by PCR using GoldStar polymerase (Eurogentec), primer Bio815 (homologous to nt 2772–2790 and containing biotin at the 5′ end), and Bio303. The resulting PCR fragment was analyzed on 1.5% agarose gel and prepared for sequencing by separation of the strands using Dyna beads according to standard procedures (Dynal). The sequences stretching from about nt 2940 to 3020 and from about nt 4000 to 4100, containing both sides of ld VIII, were determined by the dideoxynucleotide chain termination method, using the T7 sequencing kit (Pharmacia).

In vitro replication assays

Templates for measuring replication activity were made in vitro using T7 RNA polymerase. Because the standard procedure using *Sma* I linearized plasmid as template for transcription yielded a low quality and quantity of RNA, we made full-length Q_β cDNA by PCR amplification on pBRT7Q_β or its derivatives using Expand High Fidelity PCR System according to procedures recommended by the supplier (Boehringer Mannheim). The upstream primer was complementary to the T7 promoter sequence and the downstream, Bio303, to the 3′ end of Q_β. After removal of unincorporated nucleotides by Sephadex G25 filtration, PCR products were precipitated and dissolved in water. RNA synthesis from these templates was done by the T7 RNA transcription kit (Promega). Transcription mixtures were deproteinated by phenol/chloroform treatment, purified on Sephadex G50, and the RNA precipitated. Before use, the RNA was heated for 2 min at 70 °C. All RNA preparations were tested for correct length and absence of degradation by electrophoresis in denaturing 1.2% agarose gels.

Rate of initiation of replication was basically measured as described by Barrera et al. (1993). In short, reaction mixtures (8 μL) containing template RNA (15 nM) and HF (a saturating amount) were incubated in 75 mM Tris-HCl, pH 7.5, 10 mM Mg-acetate, 1 mM EDTA, 0.1 mM dithiothreitol at 37 °C for 2 min. Then, maintaining the same salt conditions, GTP (0.47 mM), ATP (0.19 mM), and replicase holoenzyme (4.6 nM) were added. The 16-μL mixtures were incubated at 37 °C for 45 s. Subsequently, CTP (0.19 mM), UTP (0.09 mM), 10 μCi [α-^{32}P] UTP (>22.2 TBq/mMol), and polyethylene sulfonate (5 μg/mL) were added. The 28-μL reaction mixtures are incubated at 37 °C for 10 min. Replication products were analyzed by electrophoresis on denaturing 4% PAGE. The efficiency of minus strand synthesis was measured as the amount of radioactivity in the corresponding band by a Betascope 603 blot analyzer (Betagen, Waltham, Massachusetts). As background, we used the lane in which the inhibitor of replication (PES) was present from the beginning.

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